NREL-Amoco CRADA Phase 3

Bench Scale Report 1.3

Fermentation of Pure Sugars by L1400 (pLNH33)

Project Title: Amoco-NREL CRADA Phase 3

Researcher: Susan Toon @ (303) 384-6141

Director of Research: George Philippidis @384-6888

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Objective

To determine the growth rate, sugar utilization, and by-product formation associated with strain L1400 (pLNH33) cultivated on xylose, glucose, and a mixture of xylose and glucose under microaerophilic batch conditions.

Materials and Methods

Inoculum Preparation

One mL from a frozen (-70°C) stock vial of L1400 (pLNH33) was inoculated into 50 mL containing 1% w/v corn steep liquor (CSL), 1% w/v yeast extract, 2% w/v peptone, and 2% w/v xylose at pH 5 in a 250-mL baffled Erlenmeyer flask. The flask was incubated at 30°C at an agitation of 150 rpm.

A 50% w/v solution of glucose, 50% w/v solution of xylose, 10% w/v solution of corn steep liquor (CSL), pH 5, and a 2% w/v yeast extract and 4% w/v peptone solution, pH 5 were prepared and filter sterilized through a 0.2 µm Nalgene filter unit. Two hundred fifty mL Erlenmeyer flasks with gas lock enclosures were autoclaved and each medium component was added according to the final required concentration of each component (see table 1). Sterile deionized water was added to each flask to make up the volume to 90 mL. A 10% v/v inoculum was transferred to each flask giving a final volume of 100 mL. Once inoculated the flasks were incubated at 30°C with an agitation of 150 rpm.

Sample protocol

Samples were taken at regular intervals and analyzed on the Yellow Springs Instrument (YSI) for ethanol and glucose. Samples were prepared for the HPLC to analyze for glucose, xylose, ethanol, glycerol, succinic acid, lactic acid, and acetic acid. Optical density (OD) was measured at 600 nm to monitor cell growth. The OD data were converted to g/L of cell mass based on a previously determined conversion factor of 0.5 g/L per OD unit for L1400 strain. The pH of each sample was monitored with a calibrated external pH meter.

Table 1: Flask component composition

Flask #	Xylose (g/L)	Glucose (g/L)	Yeast Extract (% w/v)	Peptone (% w/v)	CSL (% w/v)	Reference medium
1	30	0	1	2	0	YP
2	30	20	1	2	0	YP
3	0	20	1	2	0	YP
4	30	0	0	0	3	CSL
5	30	20	0	0	3	CSL
6	0	20	0	0	3	CSL

Results and Discussion

Figure 1 displays sugar utilization, whereas Figures 2 and 3 show the time course of cell mass and ethanol formation, respectively. Figure 3 was used to determine the growth rates of the microorganism. All growth rate values were derived from the exponential growth stage. The growth rate of L1400 (pLHN33) on xylose alone was 0.131 h^{-1} (doubling time (t_d) of 5.31 h) in YP and slightly higher at 0.142 h^{-1} (t_d = 4.87 h) in CSL. The doubling time was 2.5 (YP) to 2.1 (CSL) times faster on glucose than on xylose.

Table 2: Growth rates, doubling times and ethanol yields

Flask (#)	Growth Rate (h^{-1})		Doubling Time (h)		Ethanol Process Yield (% theoretical)	Ethanol Metabolic Yield (% theoretical)
	Glucose	Xylose	Glucose	Xylose		
1		0.131		5.311	71.1	75.4
2	0.327	0.026	2.122	26.66	80.4	81.8
3	0.337		2.055		83.9	86.2
"1+3"					78.0	80.7
4		0.142		4.874	73.1	76.9
5	0.299	0.023	2.315	30.67	76.3	78.3
6	0.309		2.245		79.9	81.3
"4+6"					76.0	78.8

As expected, the growth rate of the microorganism on glucose remained relatively constant (in the range 0.299-0.337 h^{-1}) in all four experiments, where that sugar was used (flasks 2, 3, 5, and 6).

Interestingly, a reduction is also observed in the growth rate of the yeast on glucose from 0.39 h^{-1} in the second transfer to 0.34 h^{-1} in the fourth transfer (13% decrease). The concept of "culture age" is a possible explanation. On the positive side, the rate of xylose fermentation does not seem to be affected by the cultivation on glucose. Even after five propagation cycles, xylose it taken up at 1.31-1.39 g/L h. Unfortunately, there are not enough data to determine if that is also the case with glucose utilization.

As expected, growth on glucose is faster than on xylose (1.5 to 2.5 times). The higher affinity of the microorganism for glucose can also be seen in the rates of xylose and glucose utilization. During exponential growth, ST2 utilizes glucose at a rate as high as 5.1 g/L h, whereas xylose utilization does not exceed 1.4 g/L h (Table 1). In addition, the nutrient source (medium) made a small difference in growth rate, but had no effect on xylose utilization (Table 1).

The growth rate of ST2 on glucose in YEPD (0.339-0.394 h^{-1}) is comparable to the growth rate determined for LNH33 on the same medium (0.327-0.337 h^{-1}), as measured in our previous work. However, the growth rate of ST2 on xylose in YEPD is significantly faster (0.178 h^{-1} to 0.259 h^{-1}) than that of LNH33 (0.131 h^{-1}). It should be noted that the flasks in this experiment were highly aerated to assist cell growth. For that reason the ethanol yields are fairly low, ranging between 74 and 80% of theoretical in YEPD, between 52 and 60% in YEPX, and between 42 and 50% in CSL-X (Table 2). Under anaerobic conditions, the cell mass yields would be lower and the ethanol yields higher. It is interesting to note that the ethanol yield decreased in all cases after the culture had been transferred a number of times. It was also observed that after glucose was depleted, cell density (measured as OD and DCW) continued to increase, while ethanol decreased, confirming that under aerobic conditions ethanol will be catabolized by this yeast in the absence of other carbon sources.

Table 2 clearly shows that during growth on xylose in YEPD or CSL, the majority of the carbon is being used for cell mass production. This is in direct contrast to the relatively low cell mass yields

Table 2: Calculated ethanol and cell mass yields.

Flask	Media	Ethanol Yield (% theoretical)	Ethanol Yield (g/g)	Cell Mass Yield (g/g)
1	YEPD	80.64	0.41	0.19
2	YEPD	81.46	0.42	0.13*
5	YEPD	75.48	0.39	0.20
6	YEPD	78.38	0.40	0.13*
9	YEPD	74.50	0.38	0.20
3	YEPX	59.19	0.30	0.33
7	YEPX	61.56	0.31	0.35
10	YEPX	52.35	0.27	0.36
4	CSL-X	50.09	0.26	0.27
8	CSL-X	41.76	0.21	0.33
11	CSL-X	42.69	0.22	0.31

*Cell mass and ethanol yield were calculated after 6 hours of fermentation; for all other flasks the yields were calculated after ~14 hours.

observed when the cells are cultivated in glucose. Besides cell mass and ethanol, the other by-products generated were carbon dioxide and insignificant amounts of (apparent) xylitol. Glycerol and lactic acid production were negligible, which is to be expected under highly aerated conditions. The lack of significant xylitol production indicates that ST2 is a more efficient fermenter of xylose than LNH33.

Conclusions and Recommendations:

Subculturing ST2 on glucose seems to have an effect on the subsequent growth rate on xylose, but not on the rate of xylose utilization. Growth on glucose, on the other hand, does not seem to be affected, although more data are needed. Hence, as far as the PDU operation is concerned, the duration of the seed fermentation should not be affected by cell propagation in a fill-and-draw operating environment. This is true as long as glucose is the carbon source for inoculation. The fact that xylose utilization is not affected by the number of propagation cycles means that xylose uptake can be considered quite stable throughout the SSCF operation. Finally, the difference in xylose utilization between YEPD medium and CSL was rather insignificant, strengthening our previous recommendation to proceed with CSL as the nutrient medium of choice in the SSCF process.

Raw Data

Shake Flask Experiment with L1400(LNHST2)

Determine rate of xylose utilization when cultures were previously grown on glucose

Sample	Time elapsed from t_0 (h)	Time elapsed (h)	OD (600 nm)	DCW (g/L)	ln(OD)	YSI Glucose (g/L)	LC Glucose (g/L)	YSI Ethanol (g/L)	GC Ethanol (g/L)	Xylose (g/L)	Succinic acid (g/L)	Xylitol (g/L)	Glycerol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
1	0.00	0.00	0.09		-2.40	20.90		0.01							
1	14.50	14.50	6.15	3.95	1.82	0.01	0.12	8.62	NA*		0.29	ND**	0.46	0.00	0.27
2	14.50	0.00	0.59	0.45	-0.53	21.40	20.35	0.95	NA		0.26	ND	0.54	0.17	0.54
2	17.50	3.00	2.43	2.55	0.89	15.05	15.42	3.30	NA		0.30	ND	0.62	0.14	0.61
2	20.50	6.00	6.24	3.10	1.83	0.01	0.05	9.40	NA		0.37	ND	0.59	0.10	0.26
2	25.50	11.00	7.32	4.38	1.99	0.01	0.15	9.24	NA		0.33	ND	0.57	0.00	0.23
2	39.00	24.50	11.26	6.15	2.42	0.05	0.18	7.63	NA		0.26	ND	0.23	0.00	0.09
3	14.50	0.00	0.59	0.40	-0.53			0.88	NA	20.25	0.21	ND	0.27	0.00	0.00
3	17.50	3.00	1.35	0.88	0.30			1.24	NA	19.60	0.20	ND	0.30	0.00	0.22
3	20.50	6.00	2.77	1.63	1.02			1.85	NA	17.37	0.29	ND	0.58	0.00	0.59
3	25.50	11.00	5.75	3.18	1.75			3.87	NA	11.65	0.87	ND	0.66	0.00	0.52
3	39.00	24.50	13.51	7.08	2.60			6.96	NA	0.13	1.02	ND	0.27	0.00	0.24
3	48.25	33.75	15.32		2.73			5.91	NA	0.59	1.14	ND	0.22	0.24	0.22
4	14.50	0.00	0.62	0.35	-0.48			0.88	NA	20.24	0.16	ND	0.17	1.25	0.15
4	17.50	3.00	1.30	0.83	0.26			1.15	NA	19.30	0.13	ND	0.13	1.20	0.13
4	20.50	6.00	2.54	1.43	0.93			1.90	NA	17.39	0.18	ND	0.13	0.94	0.15
4	25.50	11.00	4.89	2.65	1.59			3.97	NA	11.81	0.63	ND	0.20	0.42	0.14
4	39.00	24.50	11.12	5.40	2.41			5.59	NA	1.84	1.16	ND	0.07	0.12	0.16
4	48.25	33.75	11.29		2.42			4.56	NA	0.78	1.15	ND	0.07	0.12	0.16
5	25.25	0.00	0.62	0.48	-0.47	19.90	20.84	0.92	NA		0.21	ND	0.24	0.00	0.00
5	39.25	13.00	8.05	4.60	2.09	0.02	0.15	8.90	NA		0.33	ND	0.49	0.00	0.10
6	39.25	0.00	0.90	0.43	-0.11	20.20	20.28	1.06	NA		0.28	ND	0.60	0.19	0.60
6	42.25	3.00	2.74	1.25	1.01	14.85	14.90	3.21	NA		0.30	ND	0.68	0.15	0.62
6	45.25	6.00	6.82	3.10	1.92	0.01	0.18	9.11	NA		0.31	ND	0.62	0.11	0.41
6	48.25	9.00	7.16	3.25	1.97	0.02	0.17	9.01	NA		0.34	ND	0.60	0.00	0.31
6	62.50	23.25	10.68	4.85	2.37	0.09	0.09	7.54	NA		0.33	ND	0.46	0.00	0.27
7	39.25	0.00	0.83	0.42	-0.19			0.92	NA	20.22	0.48	ND	0.83	0.49	0.85
7	42.25	3.00	1.28	0.66	0.25			1.12	NA	19.50	0.47	ND	0.69	0.46	0.98
7	45.25	6.00	2.55	1.31	0.94			1.69	NA	17.84	0.51	ND	0.66	0.29	0.89
7	48.25	9.00	4.44	2.28	1.49			2.60	NA	14.93	0.57	ND	0.67	0.00	0.62
7	62.50	23.25	14.03	7.20	2.64			7.04	NA	0.77	0.86	ND	0.44	0.00	0.32
8	39.25	0.00	0.81	0.36	-0.21			0.98	NA	20.38	0.16	ND	0.16	1.35	0.17
8	42.25	3.00	1.23	0.58	0.21			1.10	NA	19.63	0.13	ND	0.17	1.29	0.16
8	45.25	6.00	2.37	1.12	0.86			1.56	NA	17.84	0.15	ND	0.18	1.07	0.20
8	48.25	9.00	3.89	1.85	1.36			2.46	NA	15.16	0.20	ND	0.17	0.67	0.16
8	62.50	23.25	13.46	6.39	2.60			4.82	NA	2.37	0.55	ND	0.17	0.15	0.20
9	48.75	0.00	0.61	0.30	-0.49	20.40	20.68	0.87	NA		0.21	ND	0.25	0.00	0.00
9	62.75	14.00	8.04	3.90	2.08	0.02	0.18	9.23	NA		0.37	ND	0.56	0.00	0.21
9	66.25	17.50	8.90	4.31	2.19	0.05		8.62	NA						
9	69.25	20.50	9.55	4.63	2.26	0.05	0.19	8.29	NA		0.30	ND	0.43	0.00	0.19
10	62.75	0.00	0.89	0.46	-0.11			0.92	NA	20.41	0.33	ND	0.59	0.20	0.58
10	66.25	3.50	1.49	0.76	0.40			1.23	NA						
10	69.25	6.50	3.01	1.54	1.10			1.75	NA	17.48	0.29	ND	0.59	0.00	0.61
10	74.50	11.75	6.90	3.54	1.93			4.34	NA	10.18	0.65	ND	0.60	0.00	0.46
10	88.75	26.00	14.96	7.68	2.71			6.30	NA	0.31	0.61	ND	0.30	0.00	0.27
11	62.75	0.00	0.90	0.43	-0.10			0.94	NA	20.25	0.27	ND	0.31	1.39	0.28
11	66.25	3.50	1.35	0.64	0.30			1.21	NA						
11	69.25	6.54	2.48	1.18	0.91			1.67	NA	17.76	0.15	ND	0.17	1.05	0.20
11	74.50	11.75	5.85	2.78	1.77			4.10	NA	10.96	0.63	ND	0.20	0.36	0.15
11	88.75	26.00	13.02	6.18	2.57			5.00	NA	1.62	0.78	ND	0.14	0.09	0.19

NA* Not available at time of printing

ND** Not detected by CAT task analysis